

## Supplementary Methods for:

### *Microbiome Helper: A custom and streamlined workflow for microbiome research*

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## Microbiome Amplicon Sequencing Workflow

The following detailed protocol is for the generation of paired-end sequencing reads of 16S or 18S PCR amplicons with multiple barcodes (i.e.: “indices”) on the *Illumina MiSeq* machine of length  $\approx 450$  bp (300+300 bp with  $\sim 150$  bp overlap) using v3 chemistry. It assumes an input of 380 individual samples (+4 controls = 384) in four 96-well plates (384-well plates are discouraged). Catalog numbers are given when items from specific vendors (vs. generic choices) are required.

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### REQUIRED EQUIPMENT

- Single-channel pipettes (2-1000  $\mu$ L)
- Multi-channel (8) pipette(s) (MCP; 2-100  $\mu$ L)
- Required equipment for the DNA extraction kit(s) of your choice
- PCR machine with 96-well block
- *Invitrogen Mother E-Base* (#EB-M03) for *E-Gels 96*
- Documentation system for DNA gels (SYBR Safe filter)
- Microvolume DNA fluorescence reader (such as *Invitrogen Qubit*)
- *Illumina Experiment Manager (iEM)* software
- *Illumina MiSeq* sequencer with RTA v1.17.28 / MCS v2.2 or later

### OPTIONAL/CASE-DEPENDANT EQUIPMENT

- Bead-mill / TissueLyser / Homogenizer (if using bead-based DNA extractions)
- Microvolume DNA spectrophotometer (such as *NanoDrop*; for quantifying extracts)
- Centrifuge with rotor for 96-well plates (useful for spinning down condensate)
- Standard gel electrophoresis system (for analyzing recalcitrant samples)

### REQUIRED REAGENTS & CONSUMABLES

- Pipette filter tips (p2 to p1000)
- Reagent reservoirs for MCP
- 1.5 mL Eppendorf tubes
- DNA extraction kit(s)
- 96-well thin-wall 0.2 mL PCR plates (such as *Bio-Rad* #HSP9601)
- PCR plate films (such as *Bio-Rad* #MSB1001)

- *Thermo Phusion High-Fidelity DNA Polymerase* (#F-530L) or similar
- dNTP mix (at 40 mM = 10 mM of each base)
- *Illumina* fusion primers (see below and Excel template)
- PCR-grade water
- *Invitrogen E-Gels 96 2% with SYBR Safe* (#G7208-02)
- *Invitrogen E-Gel Low Range Ladder* (#12373-031; diluted 1:1 with PCR-grade water)
- *Invitrogen SequelPrep Normalization Kit* (#A10510-01)
- *Invitrogen Qubit dsDNA HS* reagent and assay tubes (clear 0.2 mL)
- 2 N NaOH
- 200 mM Tris-HCl, pH 7
- *Illumina PhiX Control Kit v3* (#FC-110-3001)
- *Illumina MiSeq Reagent Kit v3 (600 cycle)* (#MS-102-3003)

#### OPTIONAL/CASE-DEPENDANT REAGENTS & CONSUMABLES

- Ethanol (usually a requirement for extraction/purification kits)
- Mammalian blocking primer (see **viii** below; if substantial host contamination in 18S)
- Thin-wall 0.2 mL PCR tubes or strips with caps (for re-amplifying recalcitrant samples)
- Agarose, loading buffer/stain and 100 bp ladder (for analyzing recalcitrant samples)
- PCR product purification kit (if need to concentrate final library)

#### ADVANCE PREPARATION – ORDER AND ALIQUOT PRIMERS (1 h)

- i** Use our Excel template to copy existing 16S/18S/ITS primers or to design your own custom gene primers with the proper Illumina indices and Nextera adaptor orientations. We order IDT “Ultramers” ([www.idtdna.com](http://www.idtdna.com)) for such long primers (~80-90 nt) as their coupling efficiency is one of the highest available (critical for obtaining high proportions of full-length oligos in the mix you obtain). Order the fusion primers at 4 nmole scale in deep-well plates; one set per 96-well plate, arranged as follows, leaving blank rows in between sets:

S502	S503	S505	S506	S507	S508	S510	S511	←	Forward Set 1		
S513	S515	S516	S517	S518	S520	S521	S522	←	Forward Set 2		
N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
								↖	Reverse Set 1		
N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
								↖	Reverse Set 2		

- ii Once arrived, add 400  $\mu\text{L}$  of PCR-grade water to each well containing the primers in order to reconstitute them at a concentration of 10  $\mu\text{M}$  (1/10<sup>th</sup> the typical 100  $\mu\text{M}$  working stock concentration for primers). We have found that these usually need a significant incubation time for the lyophilized pellets to re-suspend – we typically leave them overnight at 4°C.
- iii Prepare the 1  $\mu\text{M}$  working stock **Forward Set 1 Primer Plate** by pipetting 63  $\mu\text{L}$  of PCR-grade water into each well of the 96-well PCR plate from a sterile reservoir. Rotate the deep-well primer plate 90° clockwise and align it so that the 8 occupied wells (= 8 different indices) of **row 1** line up with the 8 rows of the new plate. Working by **column** and keeping the same set of tips, transfer 7  $\mu\text{L}$  of reconstituted primer into each well of each column, mixing well by pipetting. Once complete, each column of the resulting plate will have enough primer for one complete 96-well plate PCR (leaving some extra for pipetting error; 12 columns  $\times$  5  $\mu\text{L}$  = 60  $\mu\text{L}$  required). Seal the plate with PCR film and store at -20°C.
- iv Prepare the 1  $\mu\text{M}$  working stock **Forward Set 2 Primer Plate** by repeating step **iii**, but using **row 3** of the reconstituted deep-well primer plate.
- v Prepare the 1  $\mu\text{M}$  working stock **Reverse Set 1 Primer Plate** by pipetting 45  $\mu\text{L}$  of PCR-grade water into each well of the 96-well PCR plate from a sterile reservoir. Align the deep-well primer plate horizontally (normal orientation) so that the 12 occupied wells (= 12 different indices) of **row 5** line up with the 12 columns of the new plate. Working by **row** and keeping the same set of tips, transfer 5  $\mu\text{L}$  of reconstituted primer into each well of each row, mixing well by pipetting. Once complete, each row of the resulting plate will have enough primer for one complete 96-well plate PCR (leaving some extra; 8 rows  $\times$  5  $\mu\text{L}$  = 40  $\mu\text{L}$  required). Seal the plate with PCR film and store at -20°C.
- vi Prepare the 1  $\mu\text{M}$  working stock **Reverse Set 2 Primer Plate** by repeating step **v**, but using **row 7** of the reconstituted deep-well primer plate.
- vii Once all aliquoting is complete, seal the deep-well plate with PCR film and archive at -20°C until new aliquots are required (minimized freeze-thaw cycles).
- viii *Optional: For the generation of 18S V4 amplicons from microbiome samples containing substantial non-target host DNA (ex: human, mouse, etc.), order a custom PNA mammalian blocking primer (elongation arrest in the V4 region courtesy of Laura Parfrey and Matt Lemay [UBC]) with the sequence: 5'-TCTTAATCATGGCCTCAGTT-3'. Once arrived, prepare an archival stock of 100  $\mu\text{M}$  and a working stock of 10  $\mu\text{M}$  using PCR-grade water.*

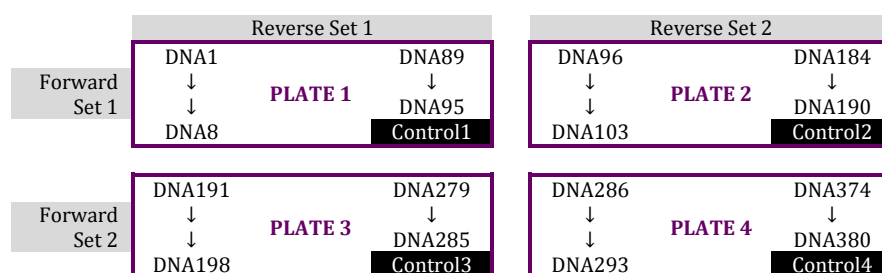
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## STEP 1 – DNA EXTRACTION (1 DAY)

- 1.1 Extract DNAs from your samples using the method/kit appropriate to the specific samples (ex: generally stool [with bead-beating kits], but also urine, etc.). For example, we have had good success with the *MO-BIO PowerFecal Kit* for mouse pellets

and human stool. *Note: Do not overload the kit columns with excessive amounts of sample material as this reduces extraction efficiencies and can allow contaminants/inhibitors to co-purify with the DNAs.*

- 1.2 Quantify and quality-check your final DNAs via *NanoDrop* or *Qubit/PicoGreen* to verify success. The A<sub>280/260</sub> ratios should be 1.8 or better and our experience with community DNAs has shown that a concentration of at least 1 ng/μL is required to get consistent PCR results. Avoid costly secondary extractions/clean-ups for inhibitors (unless obvious) until PCRs below have truly shown inhibition – many sub-optimal DNAs will still perform adequately in PCR. *Optional: A gel can be run to verify integrity (generally unnecessary for PCR-only studies).*
- 1.3 Aliquot 5 μL of each of the 380 DNA samples into 4 × 96-well PCR plates (**DNA Plates 1-4**) in the order desired (we prefer by column), allowing for one PCR negative control well (position H12) on each plate; the layout of the primers (for ex: showing F1+R2 sets are used for Plate 2) are also indicated on this figure:



## STEP 2 – 16S OR 18S PCR (1 DAY)

- 2.1 Prepare the following PCR master-mix for **Plate 1** in a 1.5 mL Eppendorf tube (adjust if not using *Phusion*):

	<b>16S or 18S standard</b>		<b>18S with blocking primer</b>	
	<b>per 25 μL rxn</b>	<b>×100 rxns</b>	<b>per 25 μL rxn</b>	<b>×100 rxns</b>
5× HF PCR Buffer	5 μL	500 μL	5 μL	500 μL
dNTPs (40 mM)	0.5 μL	50 μL	0.5 μL	50 μL
F primer (1 μM)	5 μL	} <b>added after</b>	5 μL	} <b>added after</b>
R primer (1 μM)	5 μL		5 μL	
Blocking primer (10 μM)	-	-	4 μL	400 μL
<i>Phusion</i> (2 U/μL)	0.25 μL	25 μL	0.25 μL	25 μL
PCR-grade Water	7.25 μL	725 μL	3.25 μL	325 μL
Template	2 μL	<b>added after</b>	2 μL	<b>added after</b>

- 2.2 Dispense 78.5 μL of the master-mix into the 16 wells of 2 columns (or 105 μL in 12 wells of one row) of a 96-well plate (remaining wells to be used in subsequent PCR preps) – this plate now becomes the **Master-Mix Plate** and is used to transfer the master-mix into the PCR plate using an MCP.
- 2.3 Dispense 13 μL of master-mix into each well of the **PCR Plate 1 (2 μL)**, one column (or row) at a time with the MCP.

- 2.4** Remove the protective film (“uncover”) from one **column** of the **Forward Set 1 Primer Plate**, align it horizontally on the bench to the left of **PCR Plate 1 (2 µL)** and dispense 5 µL into each well, one **column** at a time using the MCP. *Note: You can use the same set of 8 tips for all.*
- 2.5** Uncover one **row** of the **Reverse Set 1 Primer Plate**, align it vertically on the bench along the top of **PCR Plate 1 (2 µL)** and dispense 5 µL into each well, one **row** at a time using the MCP. *Note: You must now change tips after every row to avoid cross-contamination (since different F primers/indices are now in each row).*
- 2.6** Uncover the **DNA Plate 1**, align it along the top of **PCR Plate 1 (2 µL)** and dispense 2 µL into each well, one **column** at a time using the MCP. *Note: Remember to change tips after every column.*
- 2.7** Once complete, seal the plate with PCR film, place in a thermocycler and run the following program (~1.5 h; adjust if not using *Phusion*):

	<u>w/o BP</u>		<u>with BP</u>		
Initial denaturation	98°C	30 s	98°C	30 s	
Denaturation	98°C	10 s	98°C	10 s	
Blocking primer annealing	-	-	70°C	30 s	} 30 cycles
Annealing	55°C	30 s	55°C	30 s	
Extension	72°C	30 s	72°C	30 s	
Final Extension	72°C	4:30	72°C	4:30	
Hold	4°C	∞	4°C	∞	

- 2.8** While the first PCR is running, prepare the 1/10<sup>th</sup> dilution of **DNA Plate 1** – add 27 µL of PCR-grade water to each well of the remaining 3 µL of template in **DNA Plate 1**, for a total of 30 µL final volume per well, using a reservoir (~3 mL required) and MCP. *Note: Remember to change tips after every column.*
- 2.9** Once the first PCR is complete (or nearly so), repeat steps **2.1-2.7** to prepare **PCR Plate 1 (0.2 µL)** using the newly diluted templates. *Optional: Here, and for the subsequent plates below, once the two PCRs of the same plate are complete you can proceed to steps 3.1-3.4 and verify the PCR products before continuing with the next plate each time.*
- 2.10** Once the two PCRs for **Plate 1** are complete, repeat steps **2.1-2.9** to prepare **PCR Plates 2 (2 µL) & (0.2 µL)** from **DNA Plate 2** using **Forward Set 1 Primer Plate** and **Reverse Set 2 Primer Plate** (note change to F1+R2 here).
- 2.11** Once the two PCRs for **Plate 2** are complete, repeat steps **2.1-2.9** to prepare **PCR Plates 3 (2 µL) & (0.2 µL)** from **DNA Plate 3** using **Forward Set 2 Primer Plate** and **Reverse Set 1 Primer Plate** (note change to F2+R1 here).
- 2.12** Once the two PCRs for **Plate 3** are complete, repeat steps **2.1-2.9** to prepare **PCR Plates 4 (2 µL) & (0.2 µL)** from **DNA Plate 4** using **Forward Set 2 Primer Plate** and **Reverse Set 2 Primer Plate** (note change to F2+R2 here).
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### STEP 3 – GEL VERIFICATION (2 H)

- 3.1 Plug in the *Mother E-Base*, unwrap a fresh *E-Gel 96* and insert it into the base.
  - 3.2 The duplicate PCR reactions of **Plate 1** are aggregated then loaded onto the gel in the same action: using the MCP and working by **rows** (the gel cannot be loaded by columns as they are staggered), pipet 20  $\mu\text{L}$  out of the **PCR Plate 1 (0.2  $\mu\text{L}$ )** into the corresponding wells of **PCR Plate 1 (2  $\mu\text{L}$ )** and mix by pipetting up and down, then take 20  $\mu\text{L}$  of this aggregate and load into the appropriate wells of the gel. *Note: Remember to **change tips after every row**. Discard the empty **PCR Plate 1 (0.2  $\mu\text{L}$ )** when finished and relabel the **PCR Plate 1 (2  $\mu\text{L}$ )** the **Aggregated PCR Plate 1**.*
  - 3.3 Once all rows are complete, load 20  $\mu\text{L}$  of the *E-Gel Low Range Ladder* into some of the marker (“M”) wells, then run the gel for the pre-set 12 min.
  - 3.4 Visualize the gel and photograph on a UV/blue transilluminator with a SYBR filter. Example gels of 16S and 18S amplicons from this protocol are included at the end of this document for reference.
  - 3.5 Repeat steps 3.1-3.4 for **PCR Plates 2 (2  $\mu\text{L}$ ) & (0.2  $\mu\text{L}$ )**.
  - 3.6 Repeat steps 3.1-3.4 for **PCR Plates 3 (2  $\mu\text{L}$ ) & (0.2  $\mu\text{L}$ )**.
  - 3.7 Repeat steps 3.1-3.4 for **PCR Plates 4 (2  $\mu\text{L}$ ) & (0.2  $\mu\text{L}$ )**.
  - 3.8 Any samples with failed PCRs (or spurious bands) are re-amplified by optimizing the PCR (further template dilution to 1:100 or using BSA/other additives) to produce correct bands in order to complete the amplicon plate. Unless this represents the majority of a plate (in which case continue with plates and E-gels), PCRs are done in standard tubes/strips and visualized using a traditional gel box. Once correct bands have been obtained, amalgamate those few tubes into the appropriate wells of the respective **Aggregated PCR Plates** before continuing.
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### STEP 4 – PCR CLEAN-UP + NORMALIZATION & FINAL LIBRARY POOL (2 H)

- 4.1 Use the remaining 20  $\mu\text{L}$  of each well in the **Aggregated PCR Plate 1** to cleaned-up and normalize the amplicons using the high-throughput *Invitrogen SequalPrep 96-well Plate Kit*. Label this final plate **SequalPrep Plate 1**. *Note: We have found it more convenient to inverse steps 1 & 2 of the SequalPrep protocol (i.e.: buffer first with one set of tips, then amplicons with another set). Only one set of tips (per step) is need in this kit as carry-over is minimal, amplicons are barcoded (therefore can't "contaminate" each other's reads) and amplicons will shortly be pooled anyways.*
- 4.2 Once the *SequalPrep* protocol is complete, pool the 95 samples from **SequalPrep Plate 1** by using the MCP to transfer 5  $\mu\text{L}$  of each column into one column of a new 96-well plate named the **Library Pool Plate** (remaining columns to be used in subsequent pooling). *Note: You can use the same set of 8 tips for all.* Once complete, pipette 50  $\mu\text{L}$  of each of the 8 wells into one 1.5 mL Eppendorf tube and label **Plate 1 Library Pool**.

- 4.3** Repeat steps **4.1 & 4.2** for **Aggregated PCR Plate 2**.
- 4.4** Repeat steps **4.1 & 4.2** for **Aggregated PCR Plate 3**.
- 4.5** Repeat steps **4.1 & 4.2** for **Aggregated PCR Plate 4**.
- 4.6** Once all four pools are complete, pipette 100 µL of each of the four tubes into one 1.5 mL Eppendorf tube and label **Final Library Pool** (add the run name to the tube or some other identifier to keep your various pools separate).
- 4.7** Quantify the **Final Library Pool** using the *Invitrogen Qubit dsDNA HS* assay (or similar fluorescence-based alternative; 5 µL of pool to be assayed) and calculate the molar concentration using the following formula, knowing that 1 ng/µL of a 500 bp amplicon = 3.29 nM:

$$\left( \frac{500 \text{ bp}}{\text{size in bp of amplicon}} \right) \times (\text{concentration in ng/}\mu\text{L}) \times (3.29)$$

For the 16S amplicon generated using our V6-V8 primers (= 574 bp, including target region + adaptors + indices) at a concentration of 3.0 ng/µL, for example:

$$\left( \frac{500 \text{ bp}}{574 \text{ bp}} \right) \times (3.0) \times (3.29) = 8.60 \text{ nM}$$

**Note:** We have found that the anticipated 1-2 ng/µL output from the *SequalPrep* plate is near impossible to achieve; we typically see concentrations in the range of 0.3-0.9 ng/µL.

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## **STEP 5 – ILLUMINA MiSEQ SEQUENCING (~3 DAYS [ONLY ~2 H HANDS-ON AT THE START AND END])**

- 5.0** This section is based upon the following *Illumina* documents, with some small procedural changes (including using the *NextSeq* variant for sample denaturation), and the inclusion of instructions to be able to load >96 samples (i.e.: 384 combinations of indices) which are not written out by *Illumina* – familiarize yourself with these documents / have them on-hand:
- *MiSeq Reagent Kit v3 – Reagent Preparation Guide*
  - *Preparing Libraries for Sequencing on the MiSeq*
  - *Denaturing and Diluting Libraries for the NextSeq 500*
  - *MiSeq System User Guide*
- 5.1** Begin thawing the v3 *Reagent Cartridge* and tube of *HT1* as instructed. Put at 4°C when complete. *Optional: Take out the day before and thaw the reagents overnight at 4°C.*
- 5.2** While waiting, prepare the *Sample Plate* and *Sample Sheet* files that will be used to run the *MiSeq* by opening the *Illumina Experiment Manager (iEM)* software.
- 5.3** Create the *Sample Plates* first – in order to run all 384 combinations of indices, 4 separate *Sample Plates* (one per plate from our protocol above) will be required.



For the samples that were in **DNA Plate 1**:

- Choose *Nextera XT v2 (Set A)* in the *iEM* wizard.
- Give the plate a unique name (we usually use our run number and append an “A” to the end; ex.: 15A).
- Copy-and-paste the 96 sample names from your Excel sheet (after having brought the file over to the *MiSeq* via USB, Dropbox or email) into the *Plate* tab, then press *Apply Default Index Layout*. You will sometimes not see the index names show up on the top row and left-hand column of this tab, but if you switch to the *Plate Graphic* or *Table* views, they will be there correctly.
- Click on *Finish* and save the *\*A.nexxt28.plt* file in the directory of your choice.

**5.4** For the samples that were in **DNA Plate 2**, repeat step **5.3** except change to *Nextera XT v2 (Set B)* and append a “B” to the filename.

**5.5** For the samples that were in **DNA Plate 3**, repeat step **5.3** except change to *Nextera XT v2 (Set C)* and append a “C” to the filename.

**5.6** For the samples that were in **DNA Plate 4**, repeat step **5.3** except change to *Nextera XT v2 (Set D)* and append a “D” to the filename.

**5.7** Now create the *Sample Sheet* by bringing in the 4 *Sample Plates* that belong to it:

- Select *MiSeq* in the *iEM* wizard, then *Other* → *FASTQ Only*.
- Input your Reagent Cartridge barcode, select *Nextera XT v2* for the Sample Prep Kit, input your Experiment Name (we usually use our complete run name; ex.: *IMR-Run15*) and change the cycles to **301** for both reads.
- On the next screen, uncheck the *Maximize* box, choose *Select Plate* at left and navigate to and select your *\*A.nexxt28.plt* file (*Plate A*).
- Once the samples are displayed, choose *Select All + Add Selected Samples*.
- Repeat the above two steps for the remaining plate files (*Plates B/C/D*).
- The Sample Sheet Status should show as **Valid** and, if so, click *Finish* to save the file, appending the run name to the end of the filename (for the above ex.: *MSxxxxxx-600V3-Run15.csv*). We find it helpful having the run name/# when returning to the files, otherwise they are only labeled with the less-informative cartridge barcode by default. If your Sample Sheet Status is showing as **Invalid**, then it is often due to identical sample names being used in error. Once corrected, the status will update.
- It is a good idea to then simply verify that the CSV file is all correct by opening the file in *WordPad* and checking that the header information is correct (date, run name, FASTQ generation, etc.) and that you see the four sets of samples below in the [Data] section (*Plate A* samples, followed by *Plate B*, etc.).

**5.8** Prepare 0.2 N NaOH as instructed, except make 10-fold less (20 µL of 1 N NaOH + 80 µL of water).

**5.9** Use the nanomolar concentration from step **4.7** to determine whether the final amplicon library must be diluted or concentrated prior to continuing. A fixed

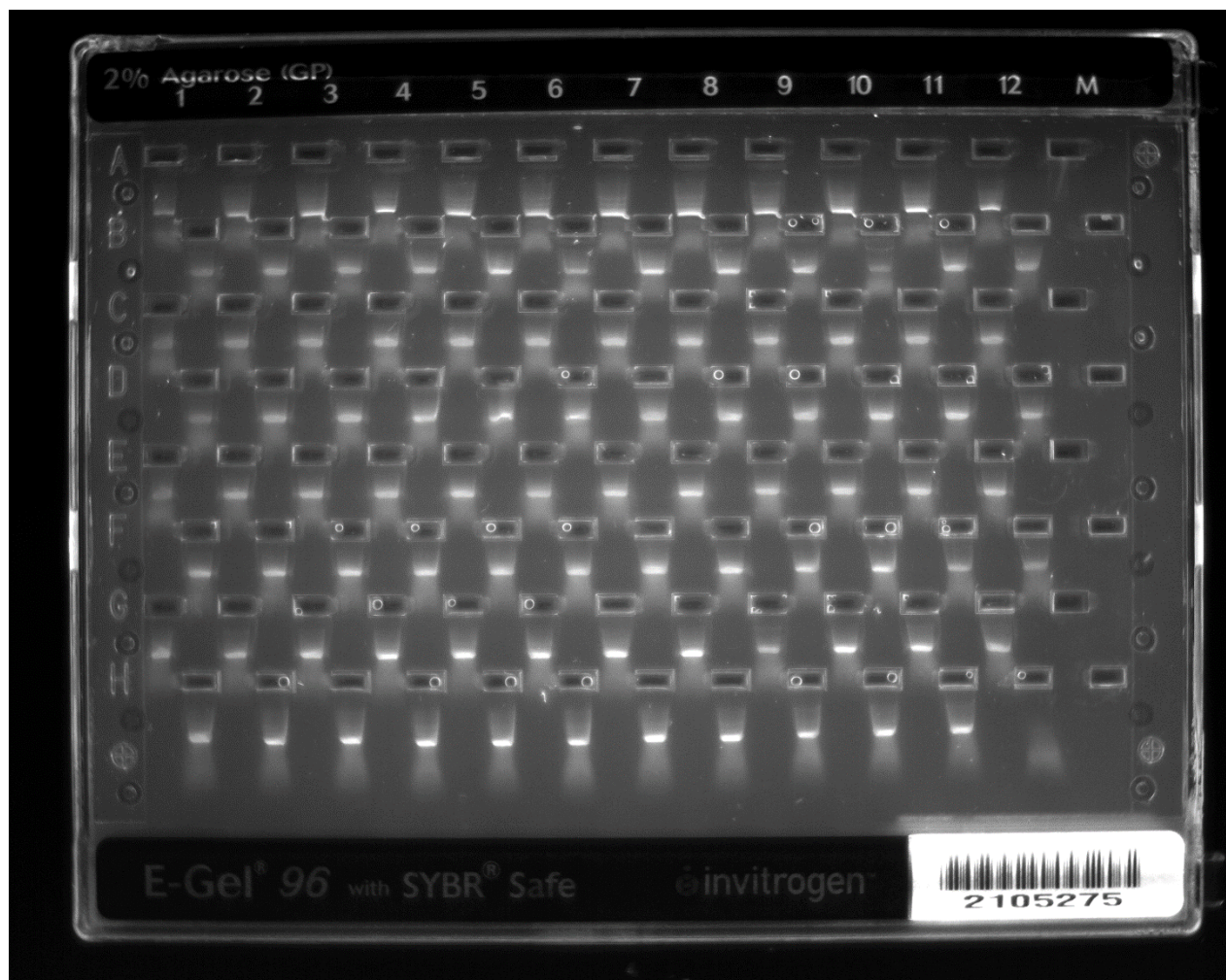


concentration of 4 nM is the standard requirement for the *MiSeq*, however, using the *NextSeq* loading protocol, a library between 0.4-4 nM can be accommodated by simply using a larger volume of a more dilute library. Use the following guidelines:

<b>[Library]</b>	<b>Vol. to denature</b>	<b>0.2 N NaOH</b>	<b>Tris-HCl</b>	<b>HT1</b>
>4 nM	<i>dilute to 4 nM using PCR-grade water or buffer</i>			
4 nM	5 µL	5 µL	5 µL	985 µL
2 nM	10 µL	10 µL	10 µL	970 µL
1 nM	20 µL	20 µL	20 µL	940 µL
0.5 nM	40 µL	40 µL	40 µL	880 µL
0.4 nM	50 µL	50 µL	50 µL	850 µL
<0.4 nM	<i>concentrate using stand. method/kit &amp; repeat step 4.7</i>			

- 5.10** Denature the library, using the indicated volume of 0.2 N NaOH in the table above, for 5 min. at room temperature.
- 5.11** Neutralize the reaction by adding the equivalent volume of Tris-HCl as indicated.
- 5.12** Dilute out the library to 20 pM using the indicated amount of chilled *HT1* and place on ice.
- 5.13** Combine 570 µL of the library with 30 µL (= 5%) of the already diluted and denatured *PhiX Control Library*. We always use 5% with amplicon libraries, regardless of anticipated diversity.
- 5.14** Proceed with loading the 600 µL sample in the *v3 Reagent Cartridge* and continue the *MiSeq* run start procedure, as instructed – the only slight change is that the default filename (just the cartridge barcode) of the *Sample Sheet* will not be found since we appended the run name to the end of the CSV file (for the above ex.: *MSxxxxxx-600V3-Run15.csv*); browse to and select the correct file.

EXAMPLE *E-GEL* 96 IMAGE OF 16S AMPLICONS FROM THIS PROTOCOL (ALL POSITIVE; *E-GEL* LOW RANGE LADDERS ABSENT FROM MARKER LANES)







EXAMPLE *E-gel* 96 IMAGE OF 18S AMPLICONS FROM THIS PROTOCOL (ALL POSITIVE; *E-gel* LOW RANGE LADDERS ABSENT FROM MARKER LANES) – NOTE THE EXTRA FAINT BANDS ABOVE THE MAIN AMPLICONS REPRESENTING THE TEMPLATES WITH INSERTIONS IN THEIR V4 REGIONS

